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Photoregulation of Cytochrome P450 Activity by Using Caged Compound

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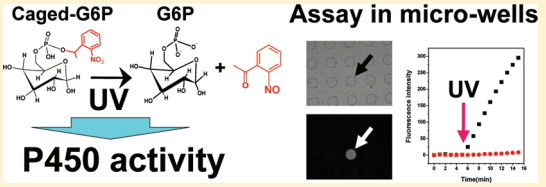
Supporting Information

ABSTRACT: Cytochrome P450 (P450) species play an important role in the metabolism of xenobiotics, and assaying the activities of P450 is important for evaluating the toxicity of chemicals in drugs and food. However, the lag time caused by the introduction and mixing of sample solutions can become sources of error as the throughput is heightened by increasing the sample number and decreasing the sample volume. To amend this technological obstacle, we developed a methodology to photoregulate the activity of P450 by using photoprotected (caged) compounds. We synthesized caged molecules of nicotinamide adenine dinucleotide phosphate ($NADP^+$) and glucose 6-phosphate (G6P), which are involved in the generation of NADPH (cofactor of P450). The use of caged-G6P completely blocked the P450 catalysis before the UV illumination, whereas caged-$NADP^+$ resulted in a little background reaction. Upon UV illumination, more than 90% of the enzymatic activity could be restored. The use of caged-G6P enabled assays in isolated microchambers (width, 50 μm; height, 50 μm) by encapsulating necessary ingredients in advance and initiating the reaction by UV illumination. The initiation of enzymatic reaction could be observed in a single microchamber. Minimizing uncertainties caused by the introduction and mixing of solutions led to significantly reduced errors of obtained kinetic constants.



We report on a methodology to regulate the activity of cytochrome P450 (P450) by photoillumination. P450 species play an important role in the metabolism of xenobiotics in organisms, and their metabolic activities in human liver are closely related with the toxicity of chemicals in drugs and food.[1,2] Methods to assay P450 activities in high throughput are sought for drug development and toxicological inspections.[3] One technological challenge in high-throughput screening is the lag time caused by the introduction and mixing of sample solutions (enzymes, substrates, cofactors, etc.), which can become sources of error in quantitative evaluation of enzymatic activities. This problem becomes critical as the volumes of solutions become smaller and the number of samples increases. One feasible approach to amend this technological obstacle is to photoregulate the enzymatic activities by using photoprotected (caged) compounds. By using a caged cofactor of the enzyme, one can mix the necessary ingredients in advance, initiate the reaction by illuminating UV light in a temporally and spatially controlled manner, and observe the reaction in real time. Although caged compounds have been extensively used *in vivo* and in time-resolved studies,[4−8] to the best of our knowledge, its application to bioassays in microchambers has not been fully exploited.

P450 requires electrons provided by P450 reductase for the enzymatic activity. P450 reductase requires, in turn, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as the electron source.[9] Therefore, it should be possible to control the enzymatic reaction by regulating the supply of NADPH. To this end, we synthesized caged molecules of nicotinamide adenine dinucleotide phosphate ($NADP^+$) and glucose 6-phosphate (G6P). $NADP^+$ can be converted into NADPH by glucose-6-phosphate dehydrogenase, consuming G6P (NADPH regeneration reaction, Figure 1). (Direct photoprotection of NADPH was not possible because of its chemical instability.) The synthesis of caged-G6P and $NADP^+$ was previously reported.[10,11] Caged-G6P and $NADP^+$ were synthesized using 2-nitrophenyl-acetophenone as the protecting group, and their abilities to photoregulate the activity of P450 were evaluated. Upon establishing the conditions for photoregulating enzymatic reactions, we conducted assays in isolated microchambers. We encapsulated all necessary ingredients, including P450 and substrate molecules, into each chamber and initiated the reaction by UV illumination. By temporally separating the mixing process and the reaction, we could observe the initial velocity of the reaction, resulting in the determination of kinetic constants with significantly smaller errors compared with the conventional methods.

MATERIALS AND METHODS

Materials. Nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phosphate

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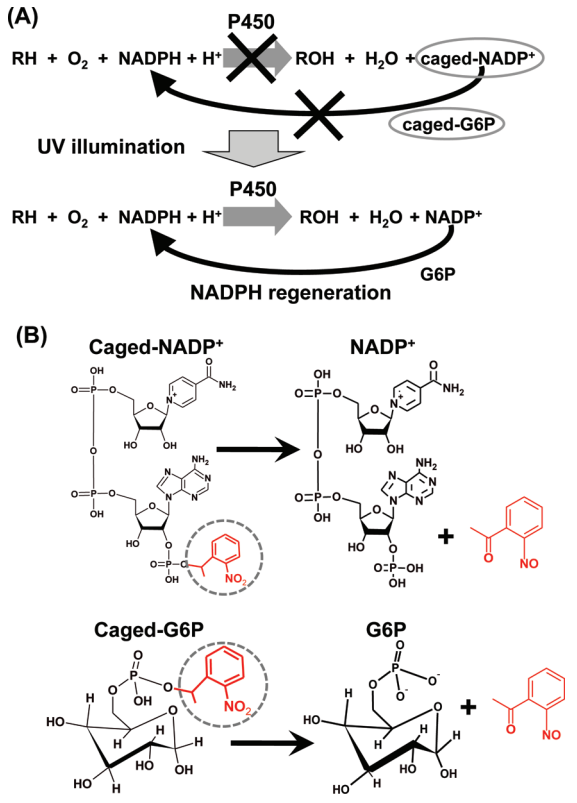

Figure 1. (A) Schematic of the photoregulation of P450 catalysis by using caged compound. (B) Chemical structure and reaction scheme of caged-NADP$^+$ and caged-G6P. The protecting groups are indicated with dotted circles.

(NADPH), dithiothreitol (DTT), magnesium chloride, and glucose-6-phosphate dipotassium (G6P) were purchased from Nacalai Tesque (Kyoto, Japan). Isopropyl-1-thio-β-D-galactopyranoside (IPTG), delta-amino levulinic acid, ethanol, methanol, and concentrated hydrochloric acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Glucose-6-phosphate dehydrogenase was purchased from Toyobo (Osaka, Japan). 7-Ethoxyresorufin (7-ER) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Milli-Q water with the resistivity more than 18 MΩ cm was used to prepare aqueous solutions.

Synthesis of Caged-NADP$^+$ and Caged-G6P. *Synthesis of Caged-NADP$^+$.* Caged-NADP$^+$ was prepared according to the reported method with some modifications.[10] A solution of 2-nitrophenyl-acetophenone hydrazone (26.9 mg, 0.15 mmol) in 0.3 mL of dichloromethane was added to manganese oxide (65.2 mg, 0.75 mmol). After stirring for 5 min at room temperature, the solution was centrifuged. The supernatant was filtered with a PTFE filter (pore diameter 0.75 μm, Millipore). The filtrate was added to 77 mg (0.1 mmol) of NADP$^+$ dissolved in 0.3 mL of deionized water and stirred for 2 h at room temperature. The aqueous phase was washed twice with dichloromethane and freeze-dried to give 116 mg of white powder. The solid was purified by C18 reverse phase high-performance liquid chromatography (HPLC) and freeze-dried to give the target caged NADP$^+$ (Figure 1). Electrospray ionization mass spectrometry (EIMS) calculated for $C_{29}H_{37}N_8O_{19}P_3$, 892.4; $[M + H^+]m/z$, 893.1.

Synthesis of Caged-G6P. Caged-G6P was prepared according to the reported method with some modifications.[12] A solution of 2-nitrophenyl-acetophenone hydrazone (225 mg, 1.26 mmol) in 1.0 mL of dichloromethane was added to manganese oxide (369.9 mg, 4.25 mmol). After stirring for 30 min at room temperature, the solution was centrifuged. The supernatant was filtered by PTFE filter (pore diameter 0.75 μm, Millipore), the filtrate was added to 87.3 mg (0.31 mmol) of glucose-6-phosphate (G6P) sodium salt dissolved in 1 mL of deionized water and stirred overnight at room temperature. The aqueous phase was washed twice with dichloromethane and freeze-dried to give 116 mg of white powder. The solid was purified by C18 reverse phase HPLC and freeze-dried twice to give the target caged-G6P (Figure 2). EIMS calculated for $C_{14}H_{21}NO_{11}P$, 409.07; $[M + Na^+]m/z$, 432.3.

Preparation of Membrane Fraction of Human P450 and P450 Reductase from *E. coli*. A pCW1A1 vector for expressing CYP1A1 in *E. coli* was constructed. The cDNA fragment encoding human CYP1A1 was obtained from human liver-cDNA libraries by using PCR. In order to modify N-terminal regions of cDNA fragments of CYP1A1 and add a digestion site by *Nde*I and *Sal*I, a primer was prepared as follows: h1A1-F, 5′-GGAATTCCATATGGCTTTTCCAATTTCAATGTCAGCAACG-3′, and h1A1-R, 5′-AAGTCGACCTAAGAGCGCAGCTGCATTTGGAAGTGCT-3′. The plasmid containing the cDNAs of CYP1A1 was digested with *Nde*I and *Sal*I, and the fragments obtained were subcloned into the *Sal*I and *Nde*I sites of the pCW vector, which contains a cDNA of human NADPH-cytochrome P450 reductase.[13] *E. coli* JM109 cells were transformed with the pCW1A1 vector. The transformed cells were cultured in 5 mL of Luria broth (LB) at 37 °C for 1 day, and then 3 mL of this culture was added to 300 mL of LB and incubated at 37 °C to an OD600 of 0.2−0.3. All liquid cultures were shook in a shaking incubator at 150 rpm. After the addition of IPTG (1 mM) and delta-amino levulinic acid (0.5 mM), the cultures were grown at 25 °C for 24 h. The cells were centrifuged and suspended in 4 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol. *E. coli* cells were homogenized by sonication. The homogenate was centrifuged at 10000*g* for 15 min to obtain a supernatant fraction, and then this supernatant was centrifuged at 100 000*g* for 60 min. Pellets (1 mL) were then resuspended in 2 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol and then stored at −80 °C until use.

P450 Assays in Test Tubes. For measuring the enzymatic activity of P450 using caged-G6P or caged-NADP$^+$ in a test tube, we used a fluorogenic substrate (7-ER). 7-ER can be dealkylated by CYP1A1 and forms a fluorescent product (resorufin). The reaction solution contained 0.1 M potassium phosphate buffer (KPB), 0.4 U/mL G6P dehydrogenase, 3 mM magnesium chloride, 1 mM DTT, 10.25 μL of P450 membrane fragments, 0.3 mM G6P (or 0.3 mM caged-G6P), and 0.01 mM NADP$^+$ (or 0.01 mM caged NADP$^+$) (total volume, 500 μL). UV illumination was conducted by using a UV lamp (Ushio, SP-7) and a bandpass filter (Schott, UG1). The applied energy was 12 mW/cm^2. Following the UV light irradiation, 1.5 μM 7-ER was added and the sample was incubated for 30 min at room temperature. The reaction was terminated by adding 25 μL of 30% trichloroacetic acid. A volume of 500 μL of chloroform was added to the reaction solution and vortexed for 1 min to extract resorufin generated by the enzymatic reaction. The organic and aqueous phases were separated by centrifugation (2 000*g* for 1 min), and 250 μL of chloroform was transferred to another tube. A volume of 500 μL of 5 mM NaOH/50 mM NaCl solution was added, and the mixture was vortexed for 1 min. The organic and aqueous phases were separated by centrifugation,

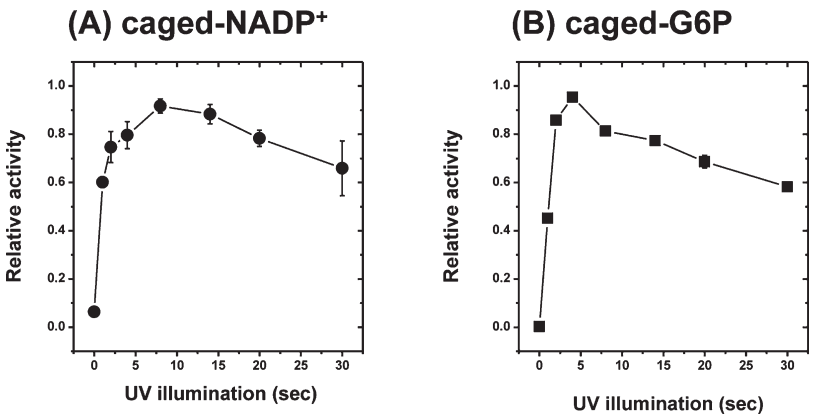

Figure 2. The enzymatic activity of h-CYP1A1 toward 7-ER, estimated from the fluorescence intensity of the product (resorufin), was plotted versus the UV dose applied to decage either caged-NADP$^+$ or caged-G6P. The measured fluorescence increase was normalized to the enzymatic reaction using normal NADP$^+$ and G6P to obtain the relative activity. The assays were conducted in a test tube by mixing the necessary ingredients (including h-CYP1A1 and a caged compound) and illuminating UV light for a defined duration. (A) caged-NADP$^+$ and normal G6P were used and (B) caged-G6P and normal NADP$^+$ were used. 7-ER was added after the UV illumination.

and 300 μL of the aqueous phase was transferred to a quartz cuvette for the fluorescence measurement (F20-SQ-5, GL Sciences, Japan). The concentration of resorufin was determined by a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) with the excitation and emission wavelength of 530 and 583 nm, respectively (Supporting Information, Figure S1). We have also confirmed by HPLC that the metabolic reaction product was resorufin (Supporting Information, Figure S2).

P450 Assays in Microwells. For P450 assays in microcompartments, a PDMS (polydimethylsiloxane) chip having microwells (diameter, 50 μm; depth, 50 μm) was used. P450 reaction solution contained 0.1 M KPB, 7-ER (varied concentrations), 0.3 mM caged-G6P, 0.4 U/mL G6P dehydrogenase, 3 mM magnesium chloride, 0.01 mM NADP$^+$, 1 mM DTT, and 10.25 μL of P450 membrane fragments (total volume, 500 μL). The solution was put on top of the PDMS chip and introduced into microwells by applying a gentle vacuum. Then the tops of the wells were sealed with a glass slide. The P450 reaction was observed by fluorescence microscopy. An upright microscope (BX51WI, Olympus, Tokyo, Japan) equipped with a xenon lamp (UXL-75XB, Olympus), a 20× water-immersion objective (NA 0.95), and a CCD camera (DP30BW, Olympus) were used. Photoactivation of caged-G6P was done by UV illumination with a bandpass filter (330−385 nm). The conversion of 7-ER into resorufin was observed with a different set of bandpass filters (excitation 545−580 nm, emission >610 nm).

RESULTS AND DISCUSSION

Photoregulation of P450 Activity Using Caged-NADP$^+$ and Caged-G6P. We first studied the photoactivation (decaging) of caged-NADP$^+$ and caged-G6P to regulate the activity of P450 enzymes. To this end, reaction solutions containing human CYP1A1 (h-CYP1A1), a caged compound (either caged-G6P or caged-NADP$^+$), and other necessary ingredients were subjected to UV illumination (Figure 2). (We have chosen h-CYP1A1 as a model P450, partially because its metabolic activity toward 7-ethoxyresorufin (7-ER) is well established.[14] It should be noted that we added 7-ER after the UV illumination in this experiment to avoid the complication caused by the photobleaching.) In the case of caged-G6P, no enzymatic reaction was observed without UV illumination. On the other hand, the use of caged-NADP$^+$ resulted in a little background reaction. It is presumably because a small amount of NADP$^+$ present can be used repeatedly by the NADPH regeneration reaction (Figure 1). For both caged-G6P and caged-NADP$^+$, the enzymatic activity increased swiftly with the UV illumination and reached maximum values, which were more than 90% of the native enzymatic activity (i.e., the enzymatic activity measured by using normal NADP$^+$ and G6P). For a longer UV illumination, the enzymatic activity gradually decreased. Since the wavelength of UV illumination overlaps the absorption band of heme in P450, a prolonged illumination may cause photodeactivation of P450 (Supporting Information, Figures S4−S6). If we used both caged-NADP$^+$ and caged-G6P for the assay, the onset of enzymatic activity occurred much more slowly, and the maximum enzymatic reaction reached was also significantly lower (Supporting Information, Figure S7). Since the use of caged-G6P could avoid the background reaction (unlike caged-NADP$^+$) and resulted in an effective switching of P450 activity, we employed it in the following experiments.

Photoregulation of P450 Activity in Microchambers. To demonstrate that initiation of enzymatic reactions can be controlled spatially and temporally, we conducted the enzymatic reaction in closed microchambers made of polydimethylsiloxane (PDMS). A reaction solution containing h-CYP1A1, substrate (7-ER), NADP$^+$, caged-G6P, and other necessary ingredients was encapsulated into PDMS microwells (diameter, 50 μm; depth, 50 μm) by putting the solution on top of the PDMS slab and evacuating gas from the microwells in vacuum. Subsequently, the wells were sealed with a glass slide. After these procedures, which took roughly 15 min, we started the assay. We could induce the enzymatic reaction in a single well by a localized UV illumination (Figure 3A). By applying an automated program comprising UV illumination and observation, we could measure the initial enzymatic reaction rate in this particular well (Figure 3B,C). No enzymatic reaction occurred in the neighboring wells (Figure 3A,B). In a different set of experiment, we measured the fluorescence increase with different 7-ER concentrations (Figure 4). From the concentration dependency of the initial velocity, we could estimate the enzymatic kinetic parameters according to the Michaelis−Menten model (Figure 5). The results are compiled in Table 1. As a comparison, we estimated the enzymatic parameters in test tubes (volume, 500 μL) using either normal G6P or caged-G6P. (It is important to note that assays in

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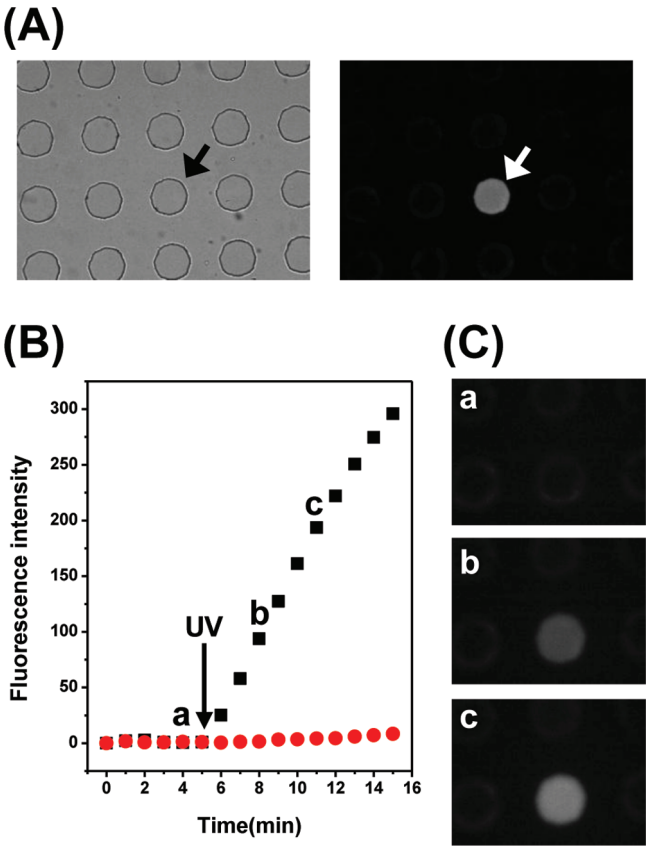

Figure 3. Spatially and temporally controlled activation of P450 in a PDMS microwell: Solutions containing h-CYP1A1, 7-ER, caged-G6P, and other necessary ingredients were encapsulated in PDMS microwells (diameter, 50 μm; height, 50 μm) and sealed with a glass slide. The enzymatic reaction in a single well was initiated by localized UV illumination using a pinhole. (A) Bright field and fluorescence images of the microwells after the reaction: Fluorescence from the reaction product (resorufin) was observed only in the illuminated microwell. (B) Time course of fluorescence increase after the illumination. Illuminated microwell showed a distinctive fluorescence increase (black squares in part B) whereas fluorescence in a neighbor chamber did not change (red circles in part B). (C) Selected fluorescence images in the time course in (B) (indicated as a, b, and c).

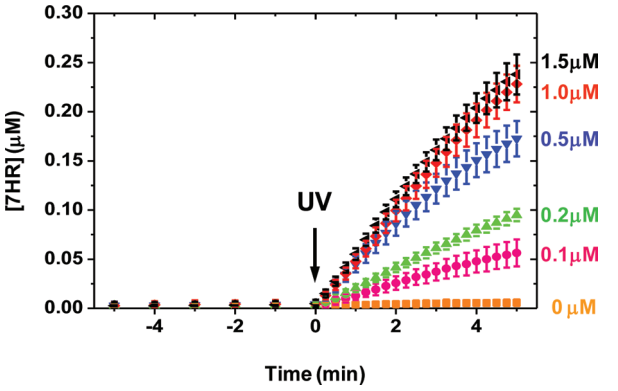

Figure 4. Assaying P450 activity in PDMS microwells: Solutions containing different concentrations of 7ER were incubated in PDMS microwells together with h-CYP1A1, caged G6P, and other necessary ingredients. The enzymatic reaction was started by UV illumination.

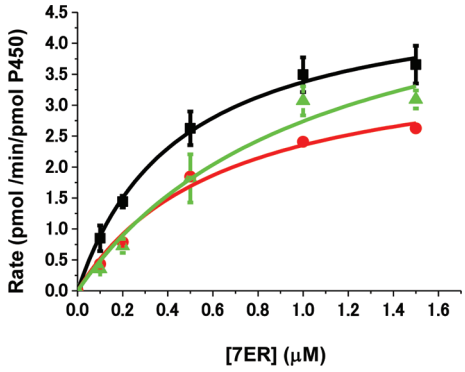

Figure 5. Initial reaction rates of h-CYP1A1 enzymatic activities were plotted versus the concentrations of 7ER to estimate the K_m and V_{max} values from the Michaelis−Menten's equation. Black square, using caged-G6P in microwells; red diamond, using caged-G6P in test tubes; green triangle, using normal G6P in test tubes.

Table 1. Enzymatic Kinetic Constants Determined by Using Normal and Caged-G6P[a]

	K_m (μM)	V_{max} (pmol/min/pmol P450)	n
caged-G6P (microwells)	0.44 ± 0.04	4.78 ± 0.35	5
caged-G6P (test tubes)	0.65 ± 0.04	3.88 ± 0.03	3
normal-G6P (test tubes)	1.11 ± 0.38	5.65 ± 0.45	3

[a] Metabolic activity of h-CYP1A1 toward 7-ER was measured in test tubes and microwells at room temperature (24 °C).

microwells using normal G6P were not possible because the enzymatic reaction started upon mixing the solutions before incorporating them into each well.) The obtained values for K_m were comparable to the literature values.[15] On the other hand, V_{max} may be slightly underestimated in the case of caged-G6P due to the effects of UV illumination. Importantly, errors of the estimated K_m and V_{max} values were significantly lower for caged-G6P compared with normal G6P (the results in test tubes). The reduced uncertainty should stem from the fact that we could measure the initial velocity of the enzymatic reaction by using caged-G6P without the effects of the mixing process.

One of the most commonly applied modes of high-throughput screening involving P450 is the competitive assay between a fluorogenic substrate and a drug candidate (or potentially toxic substance). The decrease of P450 activity toward a fluorogenic substrate in the presence of an additional substance implies that the substance is either an inhibitor or a substrate of this particular P450 species.[3] As a demonstration that we can apply caged-G6P to P450 assays in a manner relevant to the high-throughput screening setting, we conducted competitive assays in PDMS microwells. Benzo[*a*]pyrene, a carcinogenic substance, was encapsulated into each well together with h-CYP1A1 and 7-ER. As shown in Figure 6, a decrease in the metabolic reaction of h-CYP1A1 toward 7-ER was observed as the concentration of benzo[*a*]pyrene was increased, which is consistent with the report that benzo[*a*]pyrene acts as a noncompetitive inhibitor for the metabolic reaction of 7-ER by h-CYP1A1.[14] This result clearly demonstrates the feasibility to assay P450 activities using caged-G6P.

Advantages of Using Caged Compounds in Bioassays. The use of caged-G6P has two distinct advantages. First, it enables one to assay using isolated microchambers in a parallel fashion. Currently, bioassays are typically conducted using microwell

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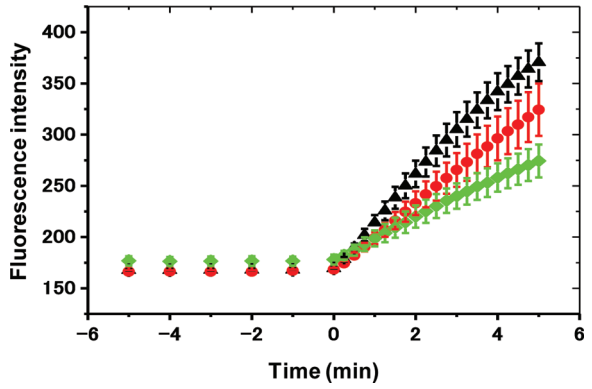

Figure 6. Competitive assay in PDMS microwells: Solutions containing 7ER and benzopyrene were incubated in PDMS microwells together with h-CYP1A1, caged G6P, and other ingredients. The enzymatic reaction was started by UV illumination at time zero. Concentration of benzopyrene: black triangles, 0 μM; red circles, 0.1 μM; green diamonds, 0.2 μM.

plates, in which each microwell has a volume of ∼10 μL. Microchambers such as demonstrated in the present study require a much smaller volume of assay solution (∼100 pL). However, bioassays in such small chambers have not been feasible because of the fact that reactions already start (and possibly finish) during the mixing and introduction of the solutions. Although femtoliter chambers have been used to study single molecule enzymatic activities,[16] the difficulty to introduce the substrate solutions into each well without starting the reaction prevented the use of isolated microchambers for high-throughput assays. The use of caged cofactors opens new possibilities to the parallel assay of enzymatic activities in isolated microchambers. One can possibly immobilize different CYPs in microwells on a chip by applying the printing technology and assay their activities by introducing the substrate solution and sealing the wells.

The second important advantage of using caged-G6P is the fact that one can measure the initial velocity of the enzymatic reaction. Kinetic models of enzymatic reactions are based on the initial velocities, but experimentally obtained initial velocities are often compromised by the lag time due to the sample introduction and mixing processes. This problem can become critical as the volume of each sample decreases and the number of samples increases. Our results suggested that we could obtain enzymatic kinetic data with a lower data fluctuation by premixing the ingredients and measuring the onset of the reaction. One potential drawback of the present approach is the possibility that enzymatic activity is affected by the UV illumination. However, we should be able to obtain accurate estimates of relative P450 activities and inhibition by applying the same decaging conditions. (Obtaining accurate values for the relative activities is often sufficient in the high-throughput screening setting because an absolute value of enzymatic activities are very difficult to attain due to many technical obstacles such as the homogeneity of P450 sources.) Furthermore, we should also be able to minimize the effects of light illumination by designing more efficient photoprotective groups (having longer absorption wavelength and higher quantum yield) and optimizing the illumination conditions.

CONCLUSIONS

We demonstrated that one can regulate P450 activity with UV illumination by using caged compounds. This resulted in two major advantages. First, we could assay P450 in isolated microchambers, drastically reducing the amount of solution necessary and increasing the number of assays that can be conducted in parallel. Second, the observation of initial velocity can minimize uncertainties caused by the introduction and mixing process of solutions, leading to significantly reduced errors of obtained assay data (kinetic constants). These features should facilitate the evaluation of P450 activities, which are important for a wide range of pharmaceutical and diagnostic applications. We have chosen h-CYP1A1 as a model P450, but we can certainly apply the same methodology to other P450 species, including h-CYP3A4, and their genetic variants. Genetic variations of P450 play important roles in the pharmacological effects of drugs to individuals. The use of caged cofactors opens a new avenue to assay a large number of P450 variants in a parallel fashion using isolated microchambers, minimizing the use of P450 enzymes and substrate materials, and improving the accuracy of assay at the same time. Furthermore, these merits of using caged cofactors can also be readily transferred to other types of clinically relevant bioassays.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Ansede, J. H.; Thakker, D. R. *J. Pharm. Sci.* **2004**, *93*, 239–255.
(2) Walsky, R. L.; Boldt, S. E. *Curr. Drug Metab.* **2008**, *9*, 928–939.
(3) Sukumaran, S. M.; Potsaid, B.; Lee, M.-Y.; Clark, D. S.; Dordick, J. S. *J. Biomol. Screen.* **2009**, *14*, 668–678.
(4) Marriott, G., Ed. *Caged Compounds*; Academic Press: London, 1998.
(5) Goeldner, M., Givens, R., Eds. *Dynamic Studies in Biology*; Wiley-VCH: Weinheim, Germany, 2005.
(6) Ellis-Davies, G. C. R. *Nat. Methods* **2007**, *4*, 619–628.
(7) Ohmuro-Matsuyama, Y.; Tatsu, Y. *Angew. Chem., Int. Ed.* **2008**, *47*, 7527–7529.
(8) Guerrero, A.; Nishigaki, T.; Carneiro, J.; Tatsu, Y.; Wood, C. D.; Darszon, A. *Dev. Biol.* **2010**, *344*, 52–65.
(9) Mueller, E. J.; Loida, P. J.; Sligar, S. G. In *Cytochrome P450*, 2nd ed.; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; pp 83−124.

(10) Cohen, B. E.; Stoddart, B. L.; Koshland, D. E., Jr. *Biochemistry* **1997**, *36*, 9035–9044.
(11) Swezey, R. R.; Epel, D. *Exp. Cell Res.* **1992**, *201*, 366–372.
(12) Swezey, R. R.; Epel, D. *Dev. Biol.* **1995**, *169*, 733–744.
(13) Uno, T.; Nakao, A.; Masuda, S.; Taniguchi, Y.; Kanamaru, K.; Yamagata, H.; Nakamura, M.; Imaishi, H.; Oono, K. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 1043–1050.
(14) Shimada, T.; Guengerich, F. P. *Chem. Res. Toxicol.* **2006**, *19*, 288–294.
(15) Liu, J.; Ericksen, S. S.; Besspiata, D.; Fisher, C. W.; Szklarz, G. D. *Drug Metab. Dispos.* **2003**, *31*, 412–420.
(16) Rondelez, Y.; Tresset, G.; Tabata, K. V.; Arata, H.; Fujita, H.; Takeuchi, S.; Noji, H. *Nat. Biotechnol.* **2005**, *23*, 361–365.